NEXT-GENERATION RECOMBINANT ANTIBODIES AND ANTIGENS FOR THE DETECTION OF BIOLOGICAL THREAT AGENTS AND SIMULANTS

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ABSTRACT

Antibodies are currently the essential component in immunological sensors that detect BW (biological warfare) agents, giving them both sensitivity and selectivity. There is considerable lot-to-lot variability in the production of antibodies. The process of developing antibodies in animals or in mammalian cell culture is also time-consuming, which limits the capacity for "just-in-time" or surge production. Powerful recombinant DNA technologies are allowing the development and eventual replacement of these traditional affinity assay reagents with novel reagents that have improved performance and ease of production. This paper describes our efforts in two such technologies, recombinant antibodies and affinity-isolated peptide aptamers.

INTRODUCTION

Advances in the ability to clone very large peptide and protein libraries and display these structures on the surfaces of bacteria and bacteriophages have led directly to their use in isolating peptides and proteins that can bind and detect BW agents and their simulants. These recombinant products can be expressed and produced in large-scale bacterial fermentations, simplifying their production over the current method of mammalian cell culture (hybridoma production of monoclonal antibodies). These recombinant methods also offer investigators the option of modifying and further improving the product through molecular evolution methods.

Advances in genetic technologies have enabled scientists to clone gene fragments encoding for antibody specificity into host expression vectors, such as bacteriophage[1,2,3] and rapidly screen the library of random gene combinations to identify those fragments of the whole antibody molecule that mediate an antibody's specific recognition of a given antigen[4,5].

RECOMBINANT ANTIBODIES

A recent advance in antibody production technology is the cloning of antibody genes and their expression in bacterial fermentations. This technology has been proven capable of producing antibodies for BW agent detection that are of higher quality and uniformity from lot to lot. Recombinant antibodies are also faster and potentially less expensive to produce and acquire in quantity; therefore, establishing a process for their production would improve the maintainability and supportability of fielded biodetection systems. We describe here the cloning

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and initial characterization of antibodies that bind the biothreat simulants bacteriophage MS2 (a non-pathogenic virus of the bacterium *Escherichia coli*, which is used to simulate viruses) and ovalbumin (a benign protein which is used to simulate protein toxins, such as ricin). To meet the need for high-quality, inexpensive antibody reagents, we have used phage display to isolate antibody genes from immunized mice. The resulting antibody molecules are called Fabs, indicating that they are comprised of heavy and light chain antibody sequences that form the antigen-binding variable region, but do not contain the IgG constant region.

Immunization, antibody gene amplification, and cloning. Antibody genes for immunoglobulin library construction were obtained from the spleens of BALB/c mice immunized with MS2 and ovalbumin. cDNA was synthesized from total spleen RNA, and amplified by PCR to isolate individual sets of immunoglobulin genes. PCR-amplified heavy chain genes are shown in Fig. 1. Heavy and light chain gene PCR fragments were assembled by ligation (MS2) or by PCR assembly (ovalbumin) and digested with restriction enzymes *Not*I and *Spe*I for cloning into the phage display vector. The primary antibody library was transformed into *E.coli* and expressed on filamentous phage particles by infection of the *E. coli* host cells with the helper phage VCSM13 (Stratagene, La Jolla, CA).

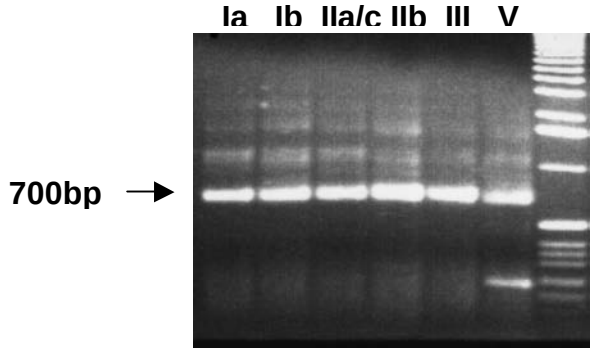

Figure 1. Amplification of heavy chain genes. Each family of mouse immunoglobulin heavy and light chain was amplified separately using PCR primers specific to that family. Shown are amplified gene fragments of six heavy chain families (Roman numerals). The desired gene fragments are 700 bp long. Other bands represent PCR artifacts. Right lane, DNA size marker.

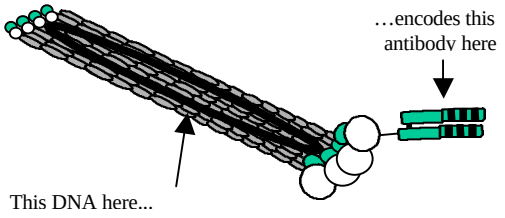

Figure 2. Schematic of antibody-displaying particle. A phage particle in the display library contains DNA encoding an antibody, and displays that same antibody on its surface. The entire repertoire of antibodies cloned from the mouse is converted to a form that can be screened by applying the population to a surface coated in the antigen of interest.

Affinity enrichment ("biopanning") of antibody clones. A purified recombinant form of the MS2 coat protein was used to screen the antibody library for clones that bind MS2. Ovalbumin for both immunization and biopanning is was obtained commercially (Pierce Co, Rockford, IL) in highly purified form. The display of the recombinant antibodies on the surface of the phage allowed for the enrichment of antigen specific clones through biopanning against

recombinant MS2 coat protein immobilized in the wells of microtiter plates. Phage particles displaying anti-MS2 or anti-ovalbumin antibodies (and containing the corresponding cloned antibody genes) were obtained by a form of affinity purification called "biopanning".

Subcloning and expression of the antibody genes. Following the last round of biopanning, individual phage clones containing the desired antibodies were identified by culturing individual clones and screening for binding of the appropriate antigen by ELISA. Verified positive clones were sequenced fully and from the sequence, the IgG subclass of each clone was determined. Genes for each Fab antibody were then removed from the phage display vectors and cloned into expression vectors that incorporate a 6x histidine tag on each heavy chain for subsequent expression and purification in fermentations of *E. coli*.

Expression and purification of recombinant anti-MS2 antibody. The introduction of antibody genes into the expression vector pHis1.1 fused the heavy chain genes with a 6xHis tag. The pHis1.1 expression vector (carrying the anti-MS2 antibody genes) was introduced into an *E. coli* strain constructed to optimize protein expression (TOPP I, Stratagene). Cells were grown in a 20-liter fermentor and the compound IPTG was added to induce expression of the antibody genes. After fermentation, cells were disrupted with a sonicator to release a crude lysate containing the recombinant anti-MS2 antibodies. The crude cell lysate was applied to a column packed with Talon metal affinity resin according to the manufacturer's instructions, and eluted with imidazole buffer. Eluted anti-MS2 antibody was further purified by passage over Sephadex gel filtration columns to greater than 90% purity. Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and HPLC for purity and protein content. Proteins were detected by staining with Coomassie Brilliant blue and by immunoblotting (Figure 4). Similar results were obtained when purifiying anti-ovalbumin antibodies (data not shown).

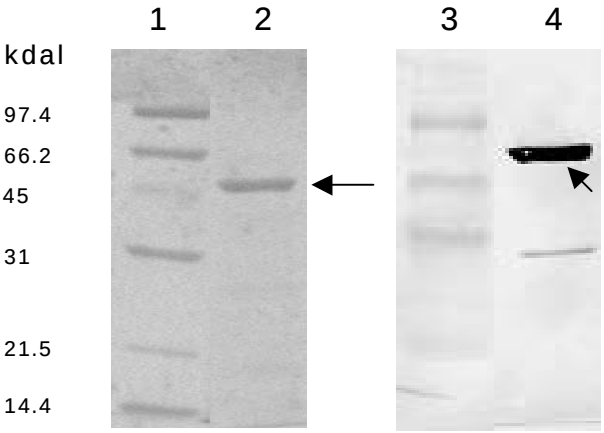

Figure 4. Purified recombinant anti-MS2 antibody. After purification as described in section 2.7, anti-MS2 antibody (lanes 2 and 4, **arrows**) was >90% pure as determined by SDS-PAGE and staining with Coomassie Brilliant blue (left image). Lane 1, molecular weight standard; lane 2, purified anti-MS2 antibody. Right image, experiment identical to lanes 1 and 2, but demonstrating the identity of the protein as mouse antibody by immunological detection. Lane 3, molecular weight marker; lane 4, purified anti-MS2 antibody. The anti-MS2 antibody was transferred to a nitrocellulose membrane after SDS-PAGE and detected with goat anti-mouse antibody conjugated to alkaline phosphatase, using NBT/BCIP as substrate.

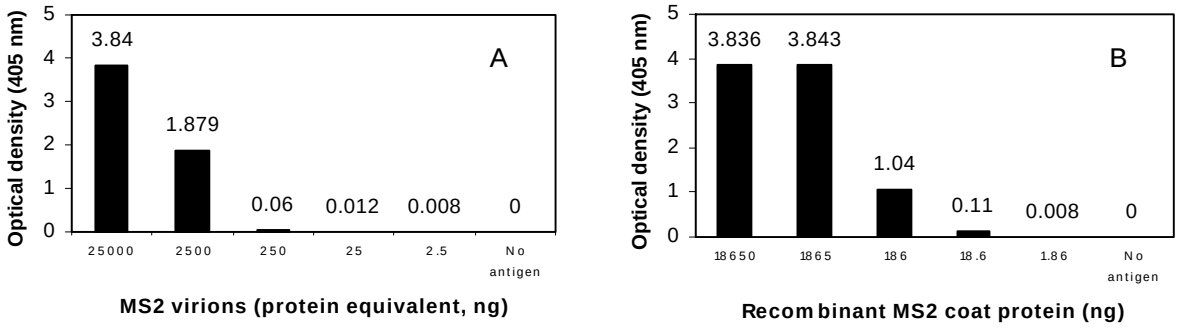

Figure 5. ELISA detection of (A) MS2 virions and (B) recombinant MS2 coat protein using purified anti-MS2 Fab antibody. Values represent the mean of 2, normalized to a no-antigen control experiment.

Detection of MS2 and ovalbumin with recombinant antibodies. Recombinant anti-MS2 and anti-ovalbumin antibodies were used in enzyme-linked immunosorbent assays (ELISAs) to detect their corresponding antigens. Density-gradient-purified bacteriophage MS2 and ovalbumin (Sigma) were serially diluted and bound by adsorption to wells of microtiter plates. Purified MS2 was detectable down to a level of 250 ng (protein equivalent) in this experiment (Figure 5). The recombinant coat protein used for biopanning and screening, however, was detected to a level of approximately 20 ng. This apparent greater sensitivity for the recombinant coat protein over the intact virus may reflect the isolation of the antibody using the recombinant protein as the biopanning target. Use of intact virions as the target, as well as performing the affinity capture of anti-body-displaying phage in solution will allow the isolation of Fabs with even greater affinity for intact MS2. Anti-MS2 did not bind phage M13, BSA, or ovalbumin (data not shown). Using partially purified recombinant antibodies OVA-3 and OVA-4, ovalbumin was detectable down to a level of 1 microgram (data not shown).

PEPTIDE-BASED BIODETECTION

Further reductionism in defining the molecular determinants of antibody specificity for antigen has been pursued by using combinatorial random peptide displays to map the specific amino acid sequences of the hypervariable region of the antibody that actually binds to the antigen epitope. It has been shown that the essential binding domains of an antibody may be as small as 5-15 amino acids[6,7,8,9]. Random peptide display libraries can be applied to two significant issues in the requirements for biodetection: 1) the design of molecular recognition elements that are smaller than whole antibodies and antibody fragments, and 2) the design of peptide sequences that can mimic the epitope binding sites on antigens and thus be used as positive controls for the antigen in assay validation. We have employed a random dodecapeptide library to screen for peptide sequences that may mimic the binding of antibody to the ricin and

staphylococcal enterotoxin B (SEB) toxins and to monoclonal antibodies generated against ricin and SEB.

Random Peptide Library. The FliTrx random peptide library was obtained commercially from Invitrogen. The library is composed of 1.77 X 10^8 primary clones of *E. coli* with the dodecamer peptide sequence inserted within the Thioredoxin (TrxA) active site loop. The TrxA peptide fusion is cloned into the nonessential domain of the major bacterial flagellin protein (FliC) under control of a P_L promoter from bacteriophage ë. When induced, the peptide sequence is expressed on the surface of the *E. coli* flagella with the N- and C-terminal ends constrained by a disulfide bond (Fig. 6).

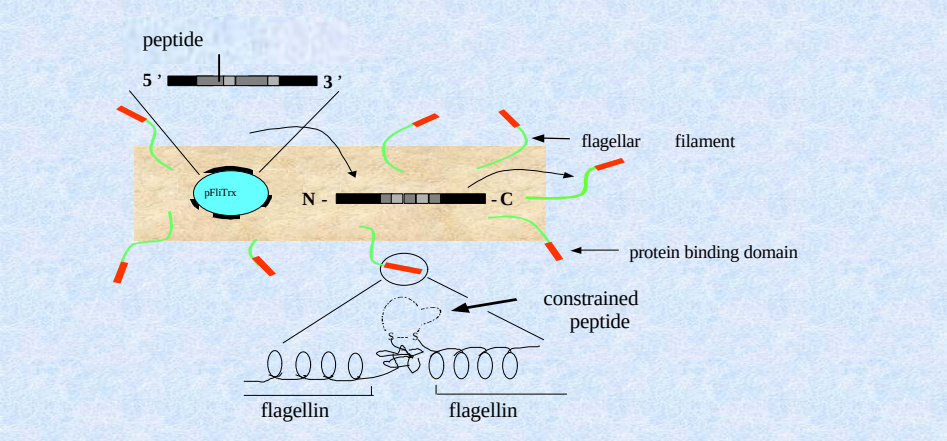

Figure 6. Schematic of peptide library construction and display.

Peptide library expression and panning. A stock culture of the cell-displayed peptide library was grown according to the manufacturer’s instructions, and expression of the peptides were induced by addition of tryptophan. The library was panned against two toxins (ricin and SEB, obtained from Sigma). Antigens were diluted and adsorbed onto tissue culture plates, rinsed and blocked. After decanting the blocking solution, the induced cell culture was added to the culture plates. Cells bound to the plate were eluted into 10 mls fresh culture media by placing the plate on a vortex to shear the flagella and release the cells to the media. The cells were then cultured overnight and the complete procedure repeated for five rounds of panning. After the fifth round of panning, culture and peptide induction, cultures expressing peptides that bound the targets were identified by competitive ELISA; antigen or antibody ligands were added to compete with the binding of the peptide clones. Individual clones that tested positive in the ELISA were selected and the peptide-encoding DNA was isolated and sequenced.

Peptides that bind SEB or ricin. Figure 8 shows the ELISA screening results obtained from peptide binding to the SEB toxin. Four of the ten clones show significant interactions with the SEB toxin, as measured by the inhibition of peptide binding in the presence of a monoclonal antibody to SEB. Four of the ten peptide clones isolated after panning against ricin show

significant activity in inhibiting the binding between ricin and a monoclonal antibody. The peptide sequences of the clones isolated did not display a consensus sequence (data not shown).

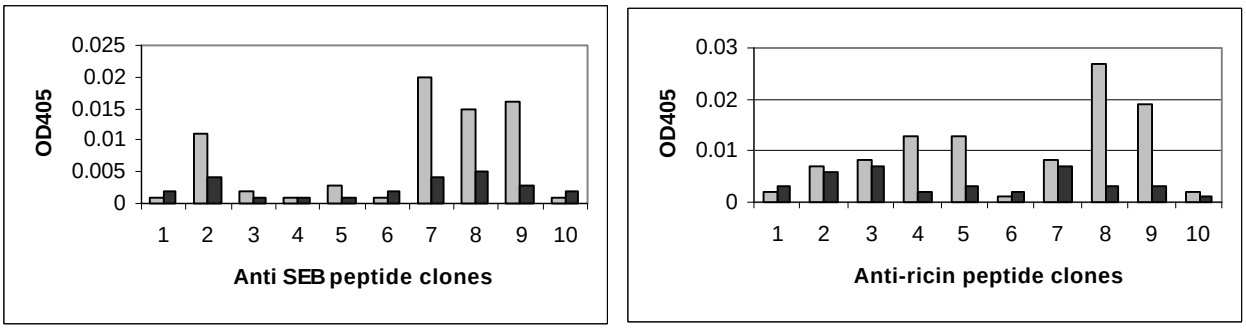

Figure 7. ELISA screening of peptide clones against SEB toxin (left) and ricin (right). Noncompetitive ELISAs are indicated by the shaded bars. Solid bars represent the inhibition of peptide-toxin interactions in the presence of anti-SEB or anti-ricin monoclonal antibody, respectively.

DISCUSSION AND CONCLUSIONS

A recombinant products approach to biodefense reagent development has several technical, logistical, and animal use advantages over traditional methods of isolating and producing antibodies. The technical advantages include the potential to produce antibody diversity greater than that obtainable by the immune systems of mammals. The biopanning procedure by its nature allows the library to self-select by allowing particles displaying a desirable antibody or peptide to bind to an immobilized target. Logistically, producing peptides and antibodies in bacterial fermentation offers several advantages. Bacteria are relatively easy to grow, and scale up in production. Their rapid growth provides the capacity for surge production. Lastly, recombinant reagent production in bacteria minimizes animal use by using only those animals initially immunized in the process. All subsequent manipulation and manufacture takes place in bacterial cells, reducing the numbers of animals used from hundreds to fewer than ten per antibody cloned.

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